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A HABA dye based colorimetric assay to detect unoccupied biotin binding sites in a fusion protein containing avidin

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We use this protocol and it's working

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Abstract

HABA (4'-hydroxyazobenzene-2-carboxylic acid) dye is an anionic dye which is used to assess the biotin binding sites in avidin. Herein we describe an assay protocol to utilize the avidin binding property of HABA to assess the number of available biotin binding sites in an avidin containing HSP70 fusion protein. This approach reduces the technical and instrumentation requirements as compared to fluorescence-based assays to evaluate biotin binding. We have also miniaturized the assay using a Nanodrop detector for the readout, thereby sparing reagents.

Attachments



[HABA dye assay narra...](#)

221KB

Materials

Reagents:

4'-hydroxyazobenzene-2-carboxylic acid (HABA) dye solution stock (10 mM)

	A	B
	HABA (Fisher Thermo Scientific Inc, Catalog number: 28010)	24.2 mg
	Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Catalog number: D8537)	9.8 mL
	1N NaOH	0.2 mL

⊗ HABA (4'-hydroxyazobenzene-2-carboxylic acid) **Thermo Fisher Catalog #28010**

⊗ Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8537**

4mM D-biotin stock solution (244.3 g/mol)

	A	B
	D-Biotin	9.8 mg
	Ultrapure water	Made up to 10 mL
	4 mM (4 mM = 0.997 mg/mL)	

20 μM Avidin (66,000 g/mol)

	A	B
	Avidin	5.3 mg
	Ultrapure water	Made up to 4 mL

⊗ Avidin from egg white **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9275-10MG**

- The Mtb HSP70-avidin fusion protein designed by Leblanc et al. (2014; Hum Vaccin Immunother 10:3022) was produced by WuXi Biologics Shanghai, China from a pool of stably transfected CHO3 cells (Ye et al., 2010;

Biotechnology Progress 26:1431).

- Four biotinylated peptides were synthesized and HPLC purified to >90% purity by 21st Century Biochemicals, Inc. Each peptide was composed of two MHC class I epitopes concatemerized with an MHC class II epitopes. The peptides were designed and biotinylated as described by Leblanc et al. (2014).

Equipment

NanoDrop Spectrophotometer

NAME

2000/2000c Spectrophotometers

TYPE

NanoDrop

BRAND

ND2000CLAPTOP

SKU

<https://www.thermofisher.com/order/catalog/product/ND2000CLAPTOP>^{LINK}



Before start

Wipe the work station with 70% Ethanol. Wear gloves before handling the proteins and dyes.

Impact of biotin concentration on the displacement of HABA from Avidin in a colorimetric assay

- 1 Prepare Avidin-HABA complex:
Add  100 μL of a  12 micromolar (μM) Avidin stock solution to a 1.7 mL eppendorf tube containing  10.4 μL DPBS. 
- 2
Add  9.6 μL of a  1 millimolar (mM) HABA stock solution to obtain a final concentration of  80 micromolar (μM) HABA dye. 
- 3 Prepare a control avidin tube lacking HABA by adding  10 micromolar (μM) avidin in a final volume of  120 μL DPBS. 
- 4 Prepare a control HABA tube by adding  9.6 μL of  1 millimolar (mM) HABA to  110.4 μL of DPBS only. 
- 5 Measure the absorbance at a wavelength of 500 nm of the HABA-Avidin complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000) by pipetting  2 μL solution onto the nanodrop pedestal. 
- 6 Prior to sample measurement, determine the spectrophotometer baseline reading using a blank solution of  2 μL DPBS. 
- 7 Add an aliquot of  10 μL of HABA-Avidin complex to each of five 0.5 mL PCR tubes. 
- 8 Add DPBS to the five tubes adequately so that after addition of D-biotin the final volume would be  15 μL . 
- 9 Add D-biotin to generate a range of D-biotin concentrations as indicated in Table 1. 

Table 1: Volumes of D-biotin and DPBS added to HABA-Avidin from stock solutions to titrate out HABA in the table below. The formula $C_1V_1=C_2V_2$ was used to determine the

volumes of D-biotin (V1) required from the stock solution (concentration C1) to achieve the final concentration (C2) in a total volume of 15 μL solution (V2).

	A	B	C	D	E
	D-biotin stock concentration (μM) C1	Final D-biotin concentration (μM) C2	D-biotin volume (μL) V1	HABA- Avidin complex volume (μL)	DPBS (μL)
	C1	4	0.75	10	4.3
	80	8	1.5	10	3.5
	80	16	3	10	2
	500	32	1	10	4
	500	64	2	10	3

Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.

Displacement of HABA from biotin binding pockets of a MtbHSP70-avidin fusion protein using different concentrations of free biotin, measured using nanodrop based detection

- 10 Prepare MtbHsp70-avidin-HABA complex:
Add  54 μL of  1.85 mg/mL a stock solution of a MtbHsp70-avidin (MAV) fusion protein to a 1.5 mL eppendorf tube containing  56.4 μL DPBS. 
- 11 Add  9.6 μL of a  1 millimolar (mM) HABA stock solution for a final concentration of  80 micromolar (μM) HABA dye and  10 micromolar (μM) concentration MAV in a final volume of  120 μL . 
- 12 Add  9.6 μL of  1 millimolar (mM) HABA to  110.4 μL of DPBS only as a control. 
- 13 Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.
- 14 Add an aliquot of  10 μL of HABA-MAV complex each of to six 0.5 mL PCR tubes. 

- 15 Add DPBS to the six tubes adequately so that after addition of biotin the total volume would be $15\ \mu\text{L}$.
- 16 Add D-biotin to the tubes to generate a range of D-biotin concentrations as indicated in Table 2.

Table 2: Volumes of D-biotin and DPBS added to HABA-MAV from stock solutions to titrate out HABA in the table below. The formula $C_1V_1=C_2V_2$ was used to determine the volumes of D-biotin (V_1) required from the stock solution (concentration C_1) to achieve the final concentration (C_2) in a total volume of $15\ \mu\text{L}$ solution (V_2).

	A	B	C	D	E
	D-biotin stock concentration (μM) C_1	Final D-biotin concentration (μM) C_2	D-biotin volume (μL) V_1	HABA- Avidin complex volume (μL)	DPBS (μL)
	40	2	0.8	10	4.2
	40	4	1.5	10	3.5
	80	8	1.5	10	3.5
	80	16	3	10	2
	200	32	2.4	10	2.6
	200	64	4.8	10	0.2

Measure the absorbance at a wavelength of 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer.

Competitive displacement of HABA by PEG4-biotinylated peptides measured in colorimetric assay

- 17 Predict the water solubility of the peptides using Pepcalc (<https://pepcalc.com>).
- 18 Dissolve the peptides to a concentration of $5\ \text{mg/mL}$ in ultrapure water with 0.5% DPBS.

Note

The HABA-MAV complex was prepared as described above.

- 19 Measure the absorbance at 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer at 500 nm wavelength.
- 20 Add an aliquot of  10 μL of HABA-MAV complex each of to six 0.5 mL PCR tubes. 
- 21 Add DPBS to the six tubes for each peptide appropriately so that after addition of peptide the total volume would be  15 μL . 
- 22 Add biotinylated peptide to the tubes to generate a range of biotinylated peptide concentrations as indicated in Table 3. 

Table 3: Volumes of biotinylated peptide and DPBS added to HABA-MAV from stock solutions to titrate out HABA in the table below. The formula $C_1V_1=C_2V_2$ was used to determine the volumes of biotinylated peptide (V_1) required from the stock solution (concentration C_1) to achieve the final concentration (C_2) in a total volume of 15 μL solution (V_2).

	A	B	C	D	E
	Biotinylated peptide stock concentration (μM) C1	Final peptide concentration (μM) C2	Biotinylated peptide stock volume (μL) V1	HABA- Avidin complex volume (μL)	DPBS (μL)
	50	4	1.2	10	3.8
	50	8	2.4	10	2.6
	50	16	4.8	10	0.2
	400	32	1.2	10	3.8
	400	64	2.4	10	2.6
	400	128	4.8	10	0.2

Measure the absorbance at 500 nm of the HABA-MAV complex and the HABA only tube using a UV-Vis Nanodrop spectrophotometer at 500 nm wavelength.

Expected results and Data Analysis

23 The calculation for this assay is based on Beer Lambert's law (Beer's law): $\Delta A = \epsilon b C$

ΔA is the difference in absorbance at 500 nm after addition of Biotin or biotin derivatives to the sample.

ϵ is the absorptivity or extinction coefficient at the wavelength (λ).

For HABA-Avidin samples at pH 7.0, the extinction coefficient at 500 nm is equal to 34,000 M⁻¹ cm⁻¹.

b is the cell path length expressed in centimeters (cm). A 10 mm-equivalent absorbance at 500 nm has a path length of 1.0 cm in Nanodrop 2000.

C is the concentration of biotin in the sample expressed in molarity (= mol/L = mmol/mL).

#Calculation 1

Absorbance at 500 nm for (HABA-avidin) reaction or (HABA-MAV) mixture = A_1

Absorbance at 500 nm for (HABA-avidin) + (biotin) reaction mixture or (HABA-MAV) + (biotin) mixture = A_2

$\Delta A = A_1 - A_2$

#Calculation 2

$C \text{ (mmol/mL)} = \Delta A / \epsilon b$

#Calculation 3

Ratio of biotin: protein

Molar concentration of bound biotin (C) / Molar concentration of original protein (MAV or Avidin)

For example,

If 10 μM Avidin is mixed with 80 μM HABA to obtain an absorbance at 500 nm, $A_1 = 1.298$, and an unknown concentration of biotin was added to the complex and absorbance at 500 nm was measured as, $A_2 = 0.04$

$\Delta A = (1.298 - 0.04)$

$\Delta A = 1.258$

$C \text{ (mmol/mL)} = \Delta A / \epsilon b$

$C = 1.258 / (34000 \times 1)$

$C = 0.000037 \text{ mmol/mL}$

$C = (0.000037 \times 1000000) \mu\text{M} = 37 \mu\text{M}$

Ratio of μmoles of biotin per μmole of Avidin = $37 \mu\text{M} / 10 \mu\text{M}$ Avidin

μmoles of biotin per μmole of Avidin = 3.7